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Original Communication

Antitumor effect of Chitosan and Silibinin and their combination in mice bearing Ehrlich ascites tumors: Impact of p53 and p21

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ABSTRACT

The main objective of the present work was to study, for the first time, the effect of naturally-derived compounds Chitosan (CS), Silibinin (SB) and their combination in different doses on the expression level of both p21 and p53 genes in Ehrlich ascites carcinoma (EAC) bearing mice considered as a model for the cancer. The aim was to evaluate the antitumor activity, lipid peroxidation, nitrosative stress, antioxidant status of CS, SB and combination treatment of both against EAC in female Swiss albino mice and to provide a first comparative assessment in this regard. **Results:** Treatment either with CS or SB alone has significantly inhibited tumor growth in a dose-dependent manner as compared to the control. Furthermore, the highest antitumor activity was resulted by SB treatment (75 mg/kg body wt) where 87.5% of treated animals showed a complete response meaning complete disappearance of tumors. The second highest antitumor activity was obtained by using combination treatment of both CS 25 mg/kg and SB 50 mg/kg where 66.67% of animals showed complete disappearance of tumors. Significant increase in superoxide dismutase (SOD) activity was observed after treatment using CS, SB and their combination. SB treatment exhibited a significant decrease in malondialdehyde (MDA) level, while no significant decrease was observed using CS alone or in combination with SB. SB (50 mg/kg) showed a significant decrease in cellular nitric oxide (NO) level; however, no significant change was observed when using CS alone or in combination with SB. Significant increase in p21 gene expression was observed using SB (50 mg/kg), CS (25 mg/kg) and combination of both. Using these doses showed synergistic additive effect. **Conclusion:** SB alone or in combination with CS was shown to be superior than CS alone, as an antitumor agent under the same experimental conditions by modulating as well as decreasing lipid peroxidation and augmenting the antioxidant defense system in EAC-bearing mice. Furthermore, antitumor effect of SB correlated with upregulated p21 gene expression and cell cycle arrest, while it had no significant effect on p53 gene expression. Finally, we suggest the therapeutic potential of using a novel combination of naturally derived compounds (CS and SB) as a target therapy for treatment of cancer; however, this combinatorial treatment deserves more investigation in the future.

KEYWORDS: Chitosan, Silibinin, cancer, Ehrlich ascites carcinoma, malondialdehyde, nitric oxide, superoxide dismutase, p21, p53

INTRODUCTION

Cancer represents the largest cause of mortality in the world, and it is estimated that 12.7 million new cancer cases occurred worldwide in 2010 [1].

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The development of an effective antitumor agent by using synthetic or naturally-occurring agents (alone or in combination with another agent) is one of the main goals in medicinal and biochemical researches. An extremely promising strategy of cancer prevention is using agents that inhibit or delay one or more stages of cancer development which in turn reduce overall cancer induction, regarded as chemoprevention of cancer. Plants, vegetables and herbs used in the folk and traditional medicine have been accepted as one of the main sources for cancer chemoprevention drug discovery and development [2]. In recent years, naturally-occurring plant products such as flavonoids as in the case of silymarin and a natural polymers such as chitosan have received much attention due to their important pharmacological properties, including antioxidant and antitumor activities [3-7]. Silymarin is a purified extract from seeds of milk thistle (Silybum marianum); it is a mixture of polyphenolic flavonoids, composed of silybin (synonymous with silibinin) as its main active component, isosilibinin, silydianin and silychristin. Silymarin extract has been empirically used as a remedy for almost 2000 years [8]. SB was found to be the major biologically active compound in silymarin with very strong antioxidant properties and a chemoprotective and canceroprotective agent, and that is why it is used as an adjuvant in the cancer static therapy [9, 10]. In terms of toxicity, SB was shown to be non-toxic and did not exhibit any side effects [11, 12].

On the other hand, the natural polymer CS is derived from chitin by N-deacetylation. It is found to have many advantageous biological properties, being biodegradable, biocompatible, non-toxic, bioabsorbable, hemostatic, bacteriostatic, fungi static, and anticholesterolemic [5]. In addition, the conjugates of some anticancer agents with CS and its derivatives showed promising anticancer efficiencies with a noticeable reduction in the undesirable side effects of the original anticancer agent [13]. El-Far *et al*. [6, 7], showed that CS and some of its derivatives significantly inhibit tumor growth using mice bearing Ehrlich ascites tumor cells. Furthermore, they showed superiority of native CS compounds over other derivatives which they prepared as antitumor agents. Their results showed that a novel water-soluble carboxymethyl chitosan-based co-polymer can induce apoptosis in EAC cells as showed by using flow cytometry cell cycle analysis, associated with a highly significant decrease in tumor volume. Their biochemical assays also revealed that treatments with CS and two prepared derivatives from it have led to an augmentation of the antioxidant defense system without affecting lipid peroxidation in EAC-bearing mice indicating their powerful antioxidant effect.

In this context, the present study was carried out to evaluate the antitumor activity, lipid peroxidation and antioxidant status of CS, SB and combinatorial treatment of both against EAC in female Swiss albino mice. Also, we examined the effect of different agents, alone or in combination, on the expression of growth-arresting and pro-apoptotic genes p53 and p21.

MATERIALS AND METHODS

Chitosan (CS) of medium molecular weight and Silibinin (SB) with Empirical formula $\mathbf{C_{25}H_{22}O_{10}}$ and molecular weight 482.44 were obtained from Sigma-Aldrich® St. Louis, MO (USA). All other reagents were of analytical grade.

Preparation of CS solution for injection

CS (250 mg) was immersed and dissolved into 10 mL of 2% (v/v) acetic acid at room temperature for 1h with stirring. The resulting acid solution was neutralized using 0.5 M (20 g/L) NaOH by its drop wise addition with stirring until turbidity appears as previously described by EL-Far *et al*. [7]. Then, the solution was diluted to desired concentrations using isotonic saline. The clear, slightly viscous and flowable solutions obtained were then adjusted to pH 6.4, which is ready for injection at desired concentrations.

Preparation of SB solution for injection

In brief, SB (120 mg) was dissolved in 10 mL of dimethyl sulfoxide (DMSO).

Animal model and transplantation of EAC cells

All experiments were performed with adult female Swiss albino mice strain purchased from Theodore Bilharz Institute, Giza, Egypt, and kept at Urology and Nephrology center, Mansoura University, Egypt, with an average body weight of 20 to 25 g. The animals

were housed in standard-size polycarbonate cages under standard laboratory conditions (26 ± 1 °C, 12-h light: 12-h dark cycle), humidity (50-60%) with food and water *ad libitum*. Mice were acclimatized to laboratory conditions for 7 days before commencement of the experiment. The study and the number of animals used were approved by the Institutional Animal Ethics Committee. The mortality was monitored for each group and recorded during the experiment. All procedures described were reviewed and approved by the University Animals Ethical Committee.

Ehrlich ascites carcinoma line was kindly supplied by the National Cancer Institute, Cairo University, Egypt. EAC cells were used for *in vivo* experiments; the tumor cell line was maintained in mice through serial intraperitoneal (i.p.) transplantations of (1×10^6) viable tumor cells in 1ml of saline using 25G needle. The tumor was characterized by moderately rapid growth, killing the mice within 3 weeks due to accumulations of ascetic fluid and showing no distal metastasis or spontaneous regression. Counting of the viable EAC cells was carried out by trypan blue exclusion method [14].

Protocol of tumor induction

130 Swiss albino female mice, one mouse per cage, were (i.p.) injected with 1 mL of EAC cells containing 1×10^6 cells for tumor induction. The day of tumor implantation was assigned as day '0'. On day 1, animals were randomized and divided into 11 different groups. Tested compounds were administered i.p. from day 1 to day 14 day after day (six separate doses). After 48 h of the last dose administration, mice were sacrificed for observation of antitumor activity, which was assessed by measuring ascetic tumor volume and cell number quantification.

The studied groups and their treatment outlines

Group 1 (n = 13): EAC-bearing mice non-treated control group: mice of this group left without any treatment until sacrifaction.

Group 2 (n = 13): EAC-bearing mice saline-treated group: animals were injected i.p. with 0.2 mL isotonic saline solution.

Group 3 (n = 16): EAC-bearing mice chitosan (25 mg/kg)-treated group: animals were injected CS solution i.p. at a dose of 25 mg/kg body weight with final volume up to 0.2 mL.

Group 4 (n = 8): EAC-bearing mice chitosan (50 mg/kg)-treated group: animals were injected CS solution i.p. at a dose of 50 mg/kg body weight with final volume up to 0.2 mL.

Group 5 (n = 12): EAC-bearing mice DMSO-treated group: animals were injected i.p. with 0.05 mL DMSO solution.

Group 6 (n = 14): EAC-bearing mice silibinin (25 mg/kg)-treated group: animals were injected i.p. with 0.05 mL SB solution (25 mg/kg body weight).

Group 7 (n = 17): EAC-bearing mice silibinin (50 mg/kg)-treated group: animals were injected i.p. with 0.05 mL SB solution (50 mg/kg body weight).

Group 8 (n = 8): EAC-bearing mice silibinin (75 mg/kg)-treated group: animals were injected i.p. with 0.05 mL SB solution (75 mg/kg body weight).

Group 9 (n = 7): EAC-bearing mice (saline + DMSO)-treated group: animals were injected i.p. with 0.2 mL isotonic saline solution and after 24 h received another 0.05 mL DMSO. This was repeated during the two weeks, which means that the animals received 6 separate doses of 0.2 mL isotonic saline and another 6 separate doses of 0.05 mL DMSO.

Group 10 (n = 7): EAC-bearing mice (CS 25 mg/kg + SB 25 mg/kg)-treated group: animals were injected i.p. with CS solution 25 mg/kg body weight with final volume of 0.2 ml. Next day, the animals were injected i.p. with SB solution (25 mg/kg body weight). This was repeated during two weeks, which means that the animals received 6 separate doses of CS solution and another 6 separate doses of SB solution.

Group 11 (n = 15): EAC-bearing mice (CS 25 mg/kg + SB 50 mg/kg)-treated group: the dose regimen of this group is the same as the previous group.

The doses of CS and SB used in this study were in the range of those used in other studies [7, 15] applied for the same animal species. They were determined after appropriate preliminary experiments.

Estimation of biochemical parameters in tumor cells

After the collection of ascetic fluids from different groups of mice, the samples were centrifuged at 1800 rpm for 5 minutes using BECKMAN GS-6R centrifuge of radius 17 cm. The supernatant was discarded. The pellets were homogenized using 5 mL

phosphate buffer saline (PBS) (pH = 7.4) per one gram cells. The homogenate was then centrifuged at 4000 rpm for 15 minutes at 4 °C. Finally, the supernatant (cell homogenate) was aspirated and stored at -80 °C until use.

Determination of Malondialdehyde (MDA) in tumor cells

Cellular levels of MDA were determined spectrophotometrically in cell homogenate according to the method of Satoh [16] using assay kit provided by Bio diagnostic, Giza, Egypt. Total protein content was determined in the samples to monitor MDA by colorimetric method according to biuret reaction, Doumas *et al*. [17] using kit provided by Eli Tech-Clinical Systems (France).

Determination of Nitric oxide (NO) as nitrosative stress biomarker in tumor cells

Cellular levels of NO were determined and estimated in cell homogenate spectrophotometrically according to the method of Montgomery and Dymock [18] using assay kit provided by Bio diagnostic, Giza, Egypt.

Determination of superoxide dismutase (SOD) activity in tumor cells

Cellular SOD activity was determined spectrophotometrically according to the method of Nishikimi *et al*. [19] using assay kit provided by Bio diagnostic, Giza, Egypt.

Quantitative, real-time polymerase chain reaction (RT-PCR)

Total RNA was isolated from (EAC) cells using a RNeasy plus Mini kit (Qiagen, USA). The yield and quality of the total RNA were determined spectrophotometrically using an absorbance of 260 and 260/280 nm ratio, respectively. The amount of RNA was quantified using a Maxima® SYBR Green/Fluorescein qPCR Master Mix (Fermentas, USA). One microgram of total RNA was reverse-transcribed into single-stranded complementary DNA (cDNA) using High Capacity RNA-to-cDNA Master Mix kit (Applied Biosystems®, USA) with a random primer hexamer in a two-step RT-PCR reaction in which any genomic DNA contamination was first eliminated using DNaseI contained in the RNeasy plus Mini kit (Qiagen, USA). The P21 and P53 mRNA levels in different EAC samples were determined using Maxima® SYBR Green/ Fluorescein qPCR Master Mix by Rotor-Gene Q (Qiagen, USA). Meanwhile, mouse Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as a housekeeping gene and an internal reference control. Gene-specific PCR primers (Table 1) were designed using Primer Express 3.0 (Applied Biosystems, USA) according to the nucleotide sequence obtained from the Gene Bank. Each primer was then blasted on NCBI/Blast to ensure its specificity to the gene required. Thermal cycling conditions included an initial activation step at 95 ºC for 10 min followed by 40 cycles of denaturation at 94 ºC for 15 seconds, annealing at 56 ºC for 30 seconds and extension at 72 ºC for one minute. Data acquisition was performed during the extension step. Melting curve analysis of the PCR product(s) was (were) performed to verify their identity and specificity. Rotor-Gene Q (Qiagen, USA) collected data automatically and analyzed the value of Threshold Cycle (Ct). Mouse P21, P53 and GAPDH mRNA relative

Table 1. The primer set used for the amplification of mouse GAPDH, P21 and P53.

Name	Sequence	Accession number	Amplicon size	Annealing temperature
Mouse GAPDH 51F	5’-ATGGTGAAGGTCGGTGTGAAC-3’	NM_008084.2	250	60 °C
Mouse GAPDH 301R	5’-TTGATGTTAGTGGGGTCTCGC-3’			
Mouse P21 239F	5’-ACGGTGGAACTTTGACTTCGTC-3’	NM_007669.4	213	60 °C
Mouse P21 452R	5’-CAGAGTGCAAGACAGCGACAAG-3’			
Mouse P53 145F	5’-CTCCGAAGACTGGATGACTGC-3’	NM_011640.3	147	60 °C
Mouse P53 292R	5’-CAACAGATCGTCCATGCAGTG-3’			

Accession number: The accession number of the sequence in the Entrez Nucleotide database

expression was determined by using the $2^{-\Delta\Delta Ct}$ method [20]. PCR products were confirmed by 1.2% agarose gel electrophoresis.

Statistical analysis

All values were expressed as mean ± S.E. The data were analyzed by ANOVA using the statistics package for Social Science software (SPSS version 15.0 Chicago, IL, USA). Differences were considered statistically significant when $p < 0.05$.

RESULTS

In vivo evaluation of the antitumor activity of different vehicles

Figure 1A demonstrates that EAC-bearing mice saline-control group showed no significant reduction in tumor volume, compared to EAC-bearing mice non-treated control group. On the other hand, EAC-bearing mice DMSO-treated group exhibited a significant reduction in tumor volume by 31.7% compared to EAC-bearing mice non-treated control group ($p < 0.05$); similar result was observed in the case EAC-bearing mice (saline + DMSO)-treated group. It exhibited a significant reduction in tumor volume by 21% compared to EAC-bearing mice non-treated control group ($p < 0.05$).

Evaluation of the antitumor activity of CS

Figure 1B demonstrates that CS injection at doses of 25 mg/kg and 50 mg/kg showed a significant decrease in tumor volume (47.5% and 73%), respectively, ($p < 0.001$) compared to the EAC-bearing mice saline-control group.

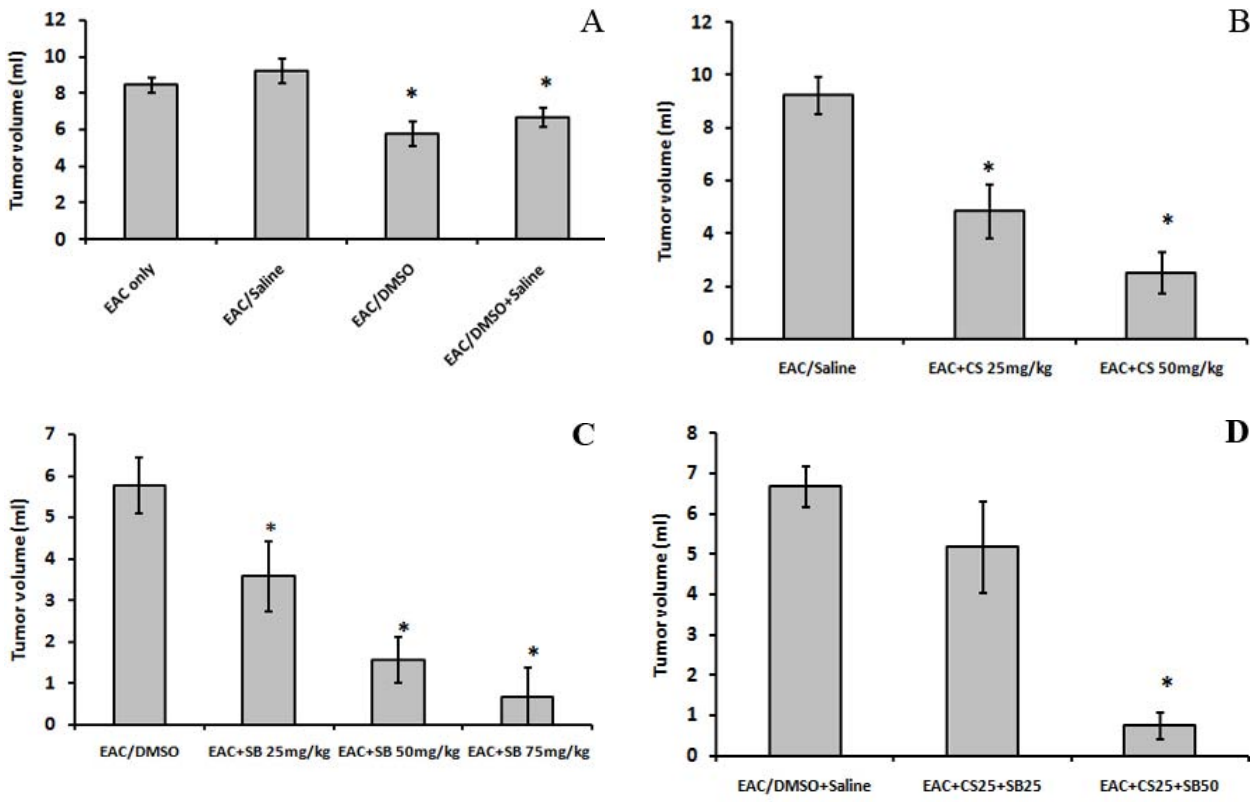

Figure 1. (A) Tumor volume in mice treated with different vehicle compared to Ehrlich ascites carcinoma (EAC) bearing mice control group (mean ± SE). **(B)** Tumor volume in mice treated with two different doses of Chitosan (CS) (25 and 50 mg/kg) compared to EAC control group (mean ± SE). **(C)** Tumor volume in mice treated with different doses of Silibinin (SB) (25, 50 and 75 mg/kg) compared to EAC control group (mean ± SE). **(D)** Tumor volume in mice treated with different doses of Chitosan (CS) and Silibinin (SB) combination (Silibinin 25 or 50 mg/kg with Chitosan 25 mg/kg) compared to EAC control group (mean ± SE). *Significant difference compared to the control group at $p < 0.05$.

Evaluation of the antitumor activity of SB

Figure 1C demonstrates that SB injection at a dose of 25 mg/kg showed a significant decrease in tumor volume by 38% ($p < 0.05$) compared to EAC-bearing mice DMSO-treated group. On the other hand, the SB at doses of 50 mg/kg and 75 mg/kg, injected to EAC bearing mice, showed significant decrease in tumor volume (73% and 88%) respectively ($p < 0.001$) compared to EAC-bearing mice DMSO-treated group. At this higher dose of SB (75 mg/kg), 7 animals showed complete response to treatment, which means no ascetic tumors observed or detected, and only one animal in this group showed a partial response to treatment with this dose (Table 2).

Evaluation of antitumor activity using combination of CS & SB

Figure 1D demonstrates that the combination of CS (25 mg/kg) with SB (25 mg/kg) showed no significant decrease in tumor volume compared to EAC-bearing mice (Saline + DMSO)-treated group. On the other hand, the combination of the same dose of CS with another higher dose of SB, i.e. (CS 25 mg/kg + SB 50 mg/kg) showed a highly significant decrease in tumor volume by 89% ($p < 0.001$) compared to EAC-bearing mice (Saline + DMSO)-treated group. Furthermore, 8 animals showed a complete response to this treatment with no ascetic tumors observed or detected, and only 4 animals in the same group showed a partial response to this treatment.

Table 2 summarizes the tumor volume in mice with respect to different treatments using CS, SB and combination of both drugs at different doses. It is obvious that SB (75 mg/kg) had the highest antitumor activity, showing complete response to treatment in 87.5% of the total treated animals under investigation. Furthermore, the combination of the same dose of CS with another higher dose of SB (CS 25 mg/kg + SB 50 mg/kg) elicited the second highest antitumor activity, showing complete response to treatment in 66.6% of the total treated animals. In contrast, SB alone (25 mg/kg) or in combination with CS at a lower dose of 25 mg/kg showed lowest antitumor activity, showing complete response to treatment in about only 17% of the total treated animals.

Effect of CS on SOD activity in tumor cells

Figure 2A demonstrates that CS injection at a dose of 25 mg/kg exhibited a significant increase ($p < 0.01$) by 43.22% in SOD activity in tumor cells compared to that of EAC-saline control group. On the other hand, treatment with CS at the dose of 50 mg/kg exhibited significant increase ($p < 0.05$) in SOD activity by 26.81% compared to EAC-bearing mice saline-treated group.

Table 2. Tumor volume in mice against different treatments.

Drugs		N (%)	Total
CS 25 mg/kg	Complete	3 (23%)	13 mice
	Partial	10 (77%)	
CS 50 mg/kg	Complete	3 (37.5%)	8 mice
	Partial	5 (62.5%)	
SB 25 mg/kg	Complete	2 (16.67%)	12 mice
	Partial	10 (83.33%)	
SB 50 mg/kg	Complete	8 (53.3%)	15 mice
	Partial	7 (46.7%)	
SB 75 mg/kg	Complete	7 (87.5%)	8 mice
	Partial	1 (12.5%)	
SB 25 mg/kg + CS 25 mg/kg	Complete	1 (16.7%)	6 mice
	Partial	5 (83.3%)	
SB 50 mg/kg + CS 25 mg/kg	Complete	8 (66.67%)	12 mice
	Partial	4 (33.33%)	

Effect of SB on SOD activity in tumor cells

Figure 2B demonstrates that SB injection at a dose of 25 mg/kg exhibited a significant increase ($p < 0.05$) in SOD activity by 48.77% in tumor target cells compared to their EAC-DMSO treated group, while SB at a dose level of 50 mg/kg also showed a significant increase ($p < 0.001$) in SOD activity by 73.68% compared to EAC-bearing mice DMSO-treated group; this value is considered the highest antioxidant activity.

Effect of combinatorial treatment of CS and SB on SOD activity in tumor cells

Figure 2C shows the effect of combination treatment of CS and SB at doses of 25 mg/kg of both on SOD activity in EAC bearing mice. This combinatorial treatment showed a significant increase ($p < 0.05$) in SOD activity by 41.21% compared to their EAC-bearing mice (Saline + DMSO)-treated control group.

Effect of CS on MDA level in tumor cells

Figure 3A shows the levels of cellular MDA in EAC bearing mice treated with different CS doses compared to their EAC-bearing mice saline-control group. As apparent, there was no significant change in the MDA level using CS at the doses of 25 mg/kg and 50 mg/kg compared to their EAC control group. However, MDA level at dose of 25 mg/kg of CS showed to be within the normal range. On the other hand, MDA at a dose of 50 mg/kg of CS showed a non-significant decreased level.

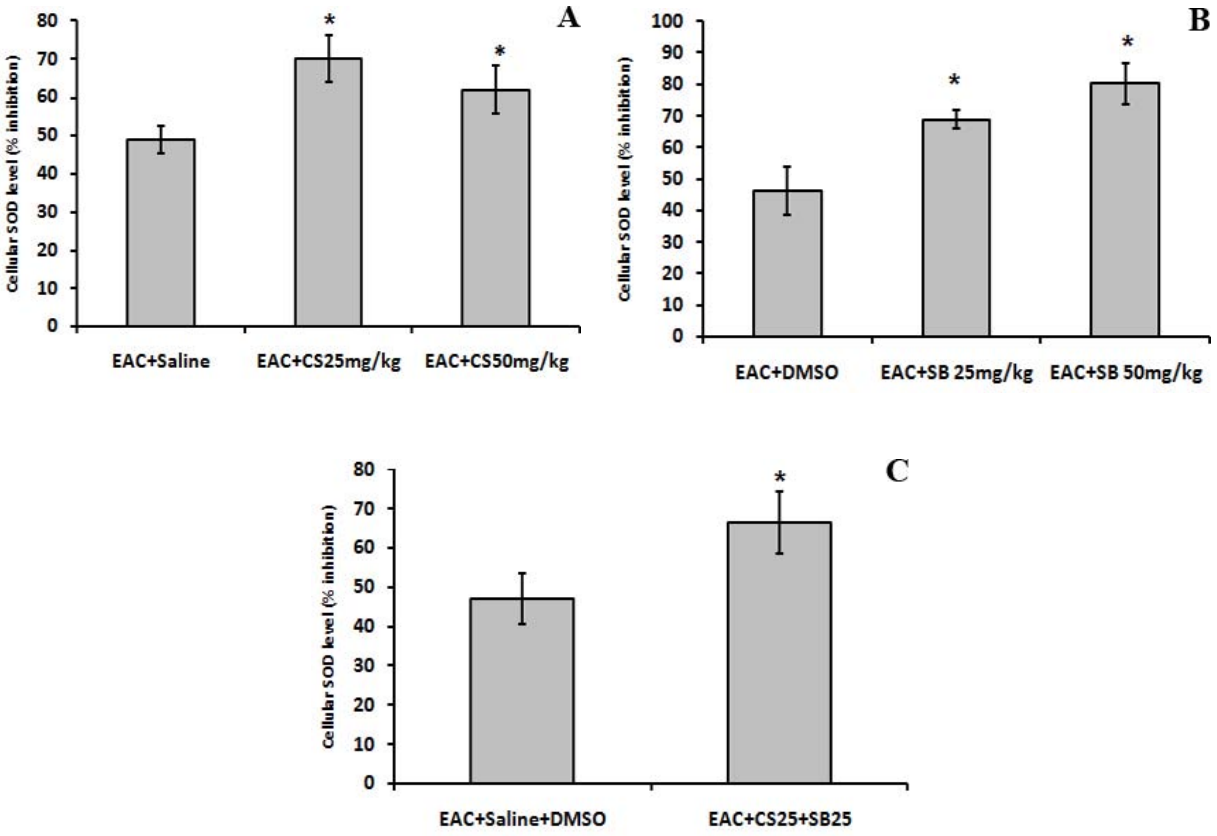

Figure 2. (A) Cellular superoxide dismutase (SOD) (% inhibition) level in mice treated with different Chitosan (CS) doses (25 and 50 mg/kg) compared to Ehrlich ascites carcinoma (EAC) bearing mice control group (mean ± SE). **(B)** Cellular SOD (% inhibition) level in mice treated with different Silibinin (SB) doses (25 and 50 mg/kg) compared to EAC control group (mean ± SE). **(C)** Cellular SOD (% inhibition) level in mice treated with Chitosan (CS) (25 mg/kg) and Silibinin (SB) (25 mg/kg) combination compared to EAC control group (mean ± SE). *significant difference compared to the control group at $p < 0.05$.

Effect of SB on MDA level in tumor cells

Figure 3B shows that SB injection at doses of 25 mg/kg and 50 mg/kg in EAC-bearing mice, exhibited a significant decrease ($p < 0.001$) in cellular MDA levels in a dose-dependent manner compared to their control group by 64.19% and 70.08%, respectively.

Effect of combinatorial treatment of CS and SB on MDA level in tumor cells

Figure 3C demonstrates that the combinatorial treatment of CS and SB at doses of 25 mg/kg of both showed a non significant decreased level of MDA by 38.21% compared to their EAC control group.

Effect of CS on NO level in tumor cells

Figure 4A demonstrates the cellular NO levels in EAC bearing mice treated with different CS doses compared to their EAC-saline control group. As apparent, CS at doses of 25 mg/kg and 50 mg/kg showed no significant change in NO level compared to the EAC-saline control group. Values found to be within normal ranges.

Effect of SB on NO level in tumor cells

Figure 4B shows that SB injection at a dose of 50 mg/kg exhibited a significant decrease ($p < 0.05$) by 69.49% compared to EAC-DMSO control group. On the other hand, SB injection at a dose of

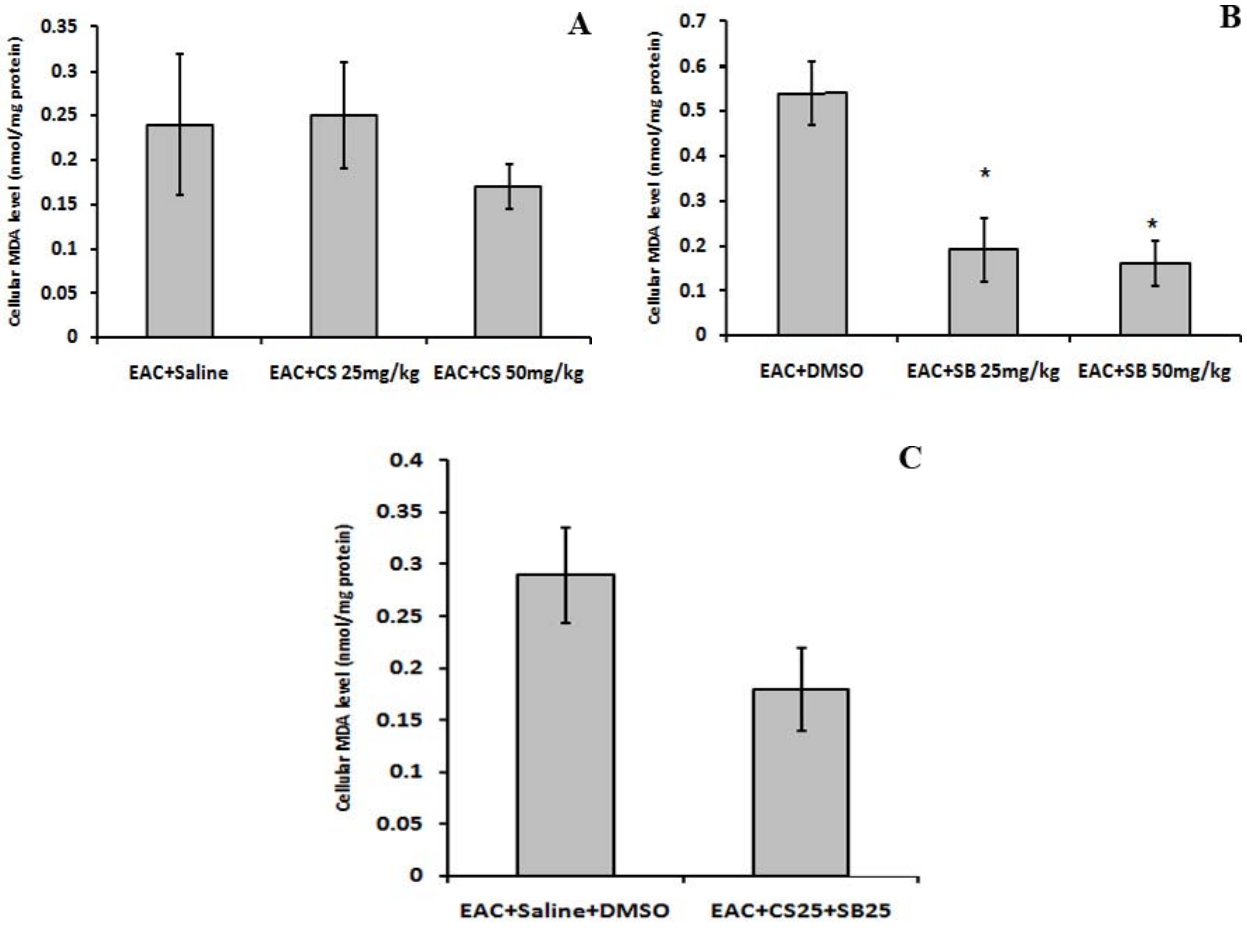

Figure 3. (A) Cellular malondialdehyde (MDA) (nmol/mg protein) level in mice treated with different Chitosan (CS) doses (25 and 50 mg/kg) compared to EAC control group (mean ± SE). **(B)** Cellular MDA (nmol/mg protein) level in mice treated with different doses of Silibinin (SB) (25 and 50 mg/kg) compared to EAC control group (mean ± SE). **(C)** Cellular MDA (nmol/mg protein) level in mice treated with Chitosan (CS) (25 mg/kg) and Silibinin (SB) (25 mg/kg) combination compared to EAC control group (mean ± SE). *significant difference compared to the control group at $p < 0.05$.

25 mg/kg showed a non-significant decrease in NO level compared to its control group.

Effect of combinatorial treatment of CS and SB on NO level in tumor cells

Figure 4C shows that combination of CS and SB at doses of 25 mg/kg of both showed a non-significant decrease in NO level by 32.01% compared to their control group.

Viable cell count results of ascetic tumors in different groups are summarized in Figure 5A, B, C and Table 3. It is in agreement with that obtained with ascetic tumor volume. Survival percentage for groups of animals under investigation is provided in Figure 6. When the mice were observed for the behavioral changes after i.p. administration of CS and SB alone, or in combination at different doses, none of the mice exhibited any abnormal behavioral responses at doses under investigation. No toxic symptoms were observed, which include inactiveness, loss of appetite or weight, slow movement, dizziness, erection of hair and hypothermia.

Effect of CS on the expression of p53 as tumor suppressor gene and p21 as cyclin dependent kinase inhibitor (CDKI)

Data in Figure 7A, B indicates that CS treatment at a dose level of 25 mg/kg exhibited a significant increase in p21 mRNA by 3.08 fold, but showed no significant effect on p53 mRNA compared to the vehicle group (saline). The use of CS at a higher dose of 50 mg/kg showed no significant effect on

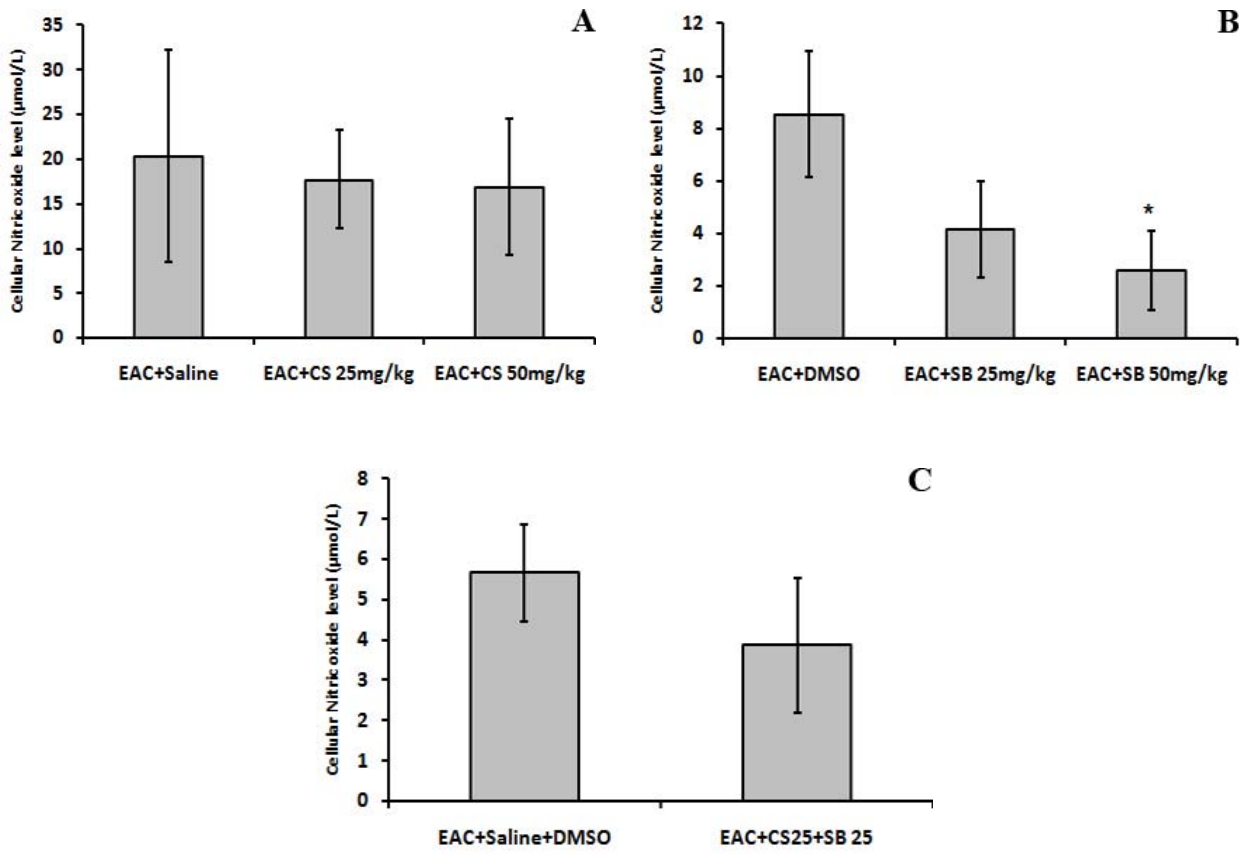

Figure 4. (A) Cellular nitric oxide (NO) (µmol/L) level in mice treated with different Chitosan (CS) doses (25 and 50 mg/kg) compared to EAC control group (mean ± SE). **(B)** Cellular nitric oxide (µmol/L) level in mice treated with different doses of Silibinin (SB) (25 and 50 mg/kg) compared to EAC control group (mean ± SE). **(C)** Cellular nitric oxide (µmol/L) level in mice treated with Chitosan (CS) (25 mg/kg) and Silibinin (SB) (25 mg/kg) combination compared to EAC control group (mean ± SE). *significant difference compared to the control group at $p < 0.05$.

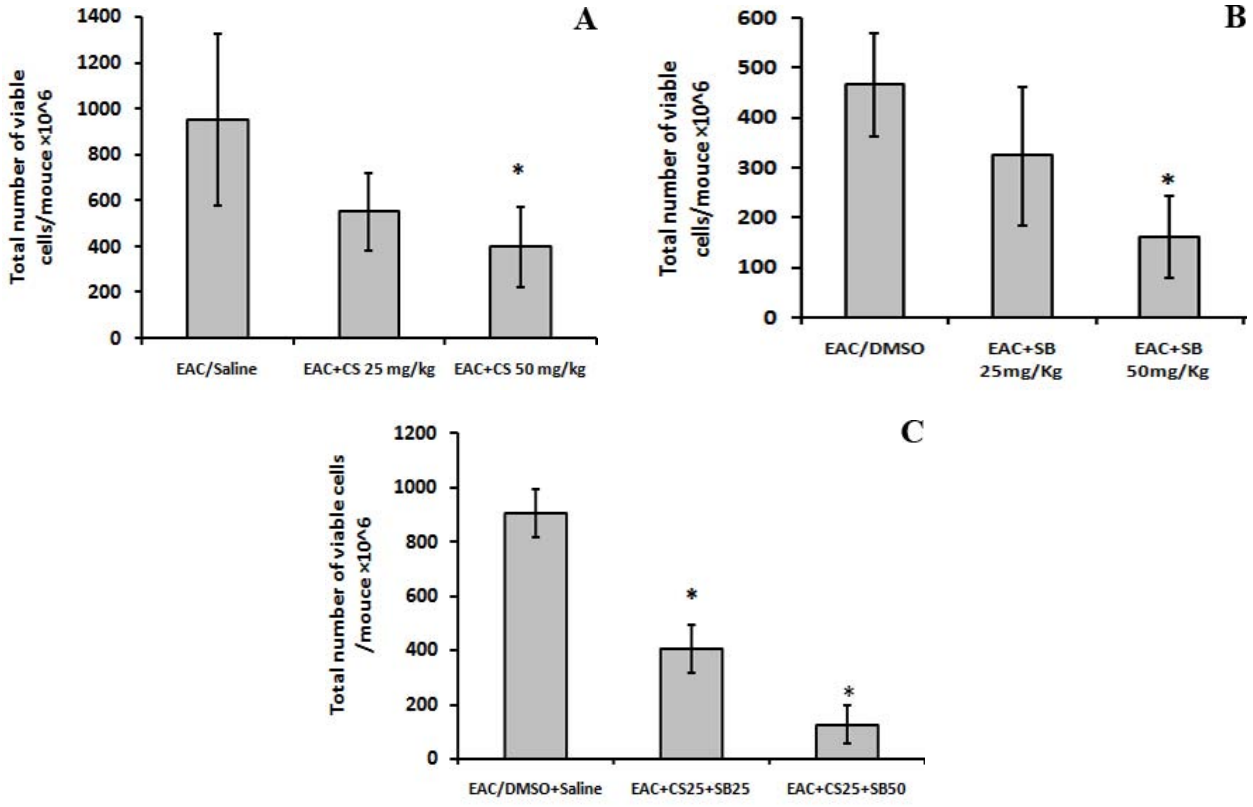

Figure 5. (A) Total number of viable cells in mice treated with different doses of Chitosan (CS) (25 and 50 mg/kg) compared to EAC control group (mean ± SE). **(B)** Total number of viable cells in mice treated with different doses of Silibinin (SB) (25 and 50 mg/kg) compared to EAC control group (mean ± SE). **(C)** Total number of viable cells in mice treated with different doses of Chitosan (CS) and Silibinin (SB) combination (Silibinin 25 or 50 mg/kg with Chitosan 25 mg/kg) compared to EAC control group (mean ± SE).*significant difference compared to the control group at $p < 0.05$

Table 3. Total number of viable cells in mice against different treatments.

Drugs		N (%)	Total
CS 25 mg/kg	Complete	3 (23%)	13 mice
	Partial	10 (77%)	
CS 50 mg/kg	Complete	3 (37.5%)	8 mice
	Partial	5 (62.5%)	
SB 25 mg/kg	Complete	2 (18.2%)	11 mice
	Partial	9 (81.8%)	
SB 50 mg/kg	Complete	8 (53.3%)	15 mice
	Partial	7 (46.7%)	
SB 75 mg/kg	Complete	7 (87.5%)	8 mice
	Partial	1 (12.5%)	
SB 25 mg/kg + CS 25 mg/kg	Complete	1 (16.7%)	6 mice
	Partial	5 (83.3%)	
SB 50 mg/kg + CS 25 mg/kg	Complete	8 (66.67%)	12 mice
	Partial	4 (33.3%)	

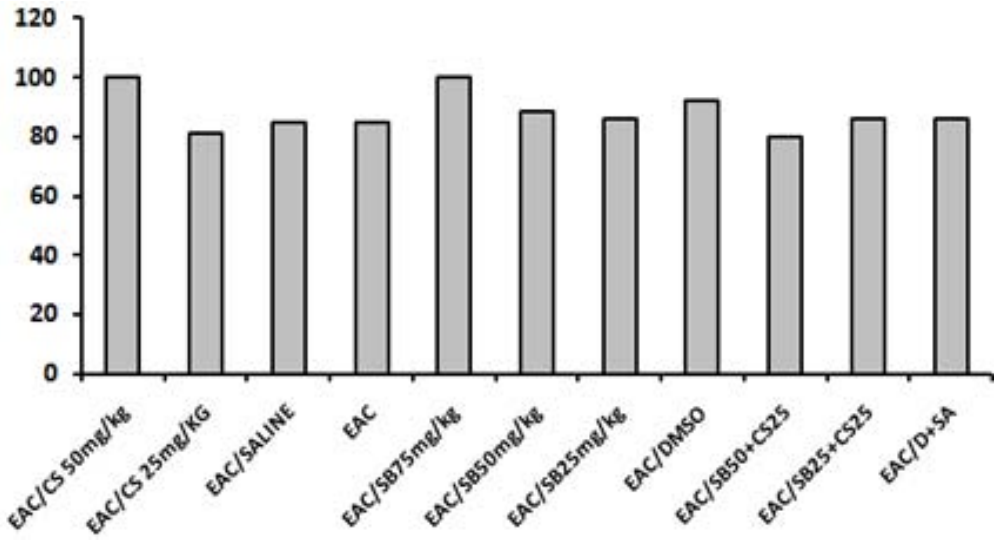

Figure 6. Survival percentage in different groups.

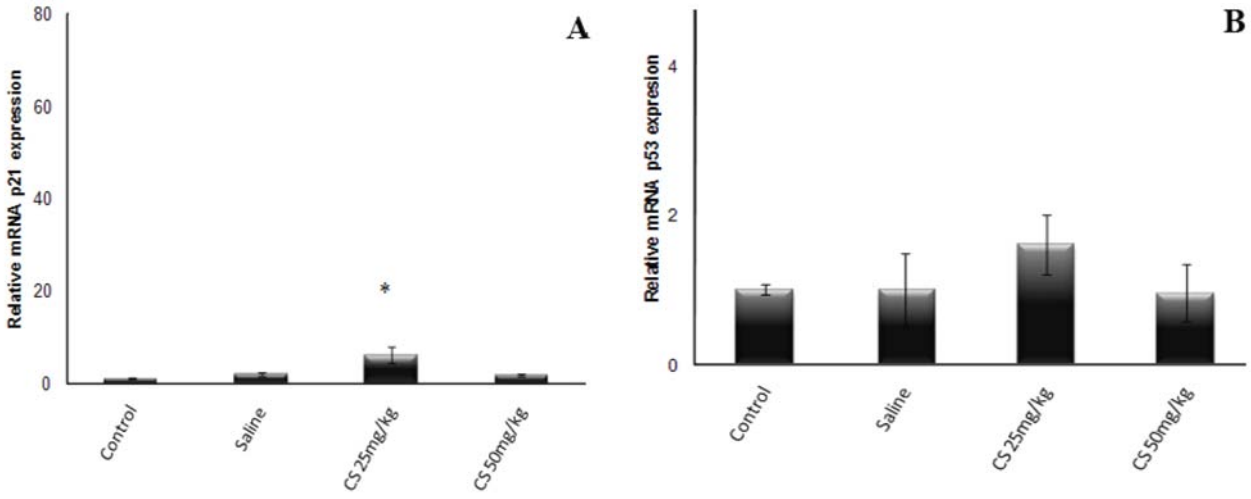

Figure 7. (A) Chitosan increases p21 expression in EAC mice model (3.08 fold at a dose of 25 mg/kg chitosan). *Significant difference compared to the saline treated groups at $p < 0.05$. **(B)** Chitosan (at different doses 25 & 50 mg/kg) has no significant effect on p53 expression.

both p53 and p21 mRNA compared to the vehicle group (saline). This figure also demonstrates that saline showed no significant effect on both p21 and p53 mRNA compared to EAC bearing non-treated control group.

Effect of SB on the expression level of p53 and p21 genes

Our data have shown that SB injection showed tumor growth inhibition in EAC model. In our attempt to unveil the mechanism of EAC tumor suppression, we examined whether or not SB showed any effect on the expression of growth-arresting and pro-apoptotic genes, p53 and p21 using real time quantitative RT-PCR. We found that SB at lower dose (25 mg/kg) showed no significant effect on both p53 and p21 expression compared to the vehicle group as shown in Figure 8A, B. The higher dose of SB (50 mg/kg) treatment exhibited a significant increase in p21 expression level by

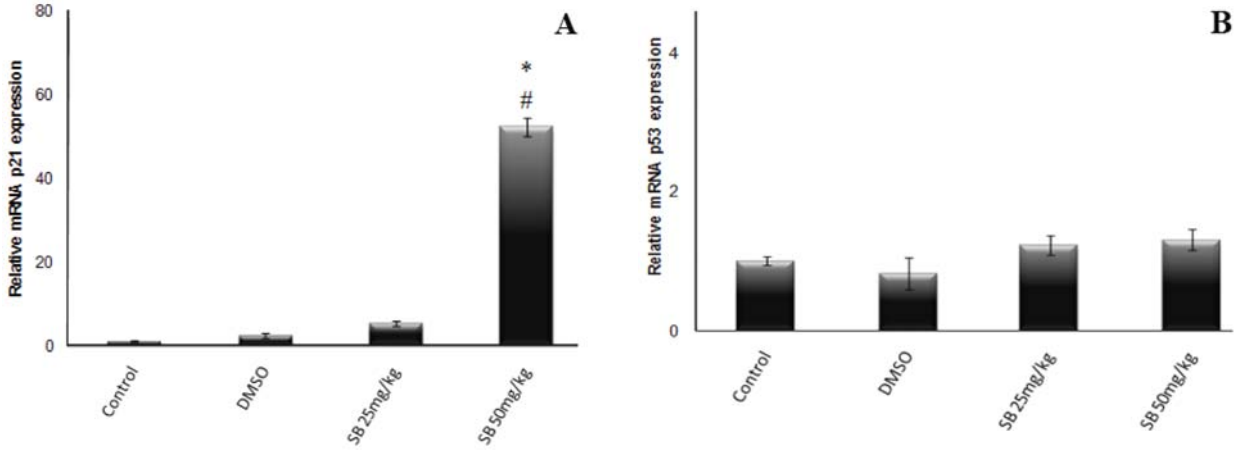

Figure 8. (A) Silibinin (SB) increases p21 expression in a dose-dependent manner in EAC mice model (22.23 fold increase at a dose of 50 mg/kg silibinin) *Significant difference compared to Dimethyl sulfoxide (DMSO) treated groups at $p < 0.001$. #Significant difference compared to silibinin at 25 mg/kg groups at $p < 0.001$. **(B)** Silibinin (at different doses 25 & 50 mg/kg) has no significant effect on p53 expression.

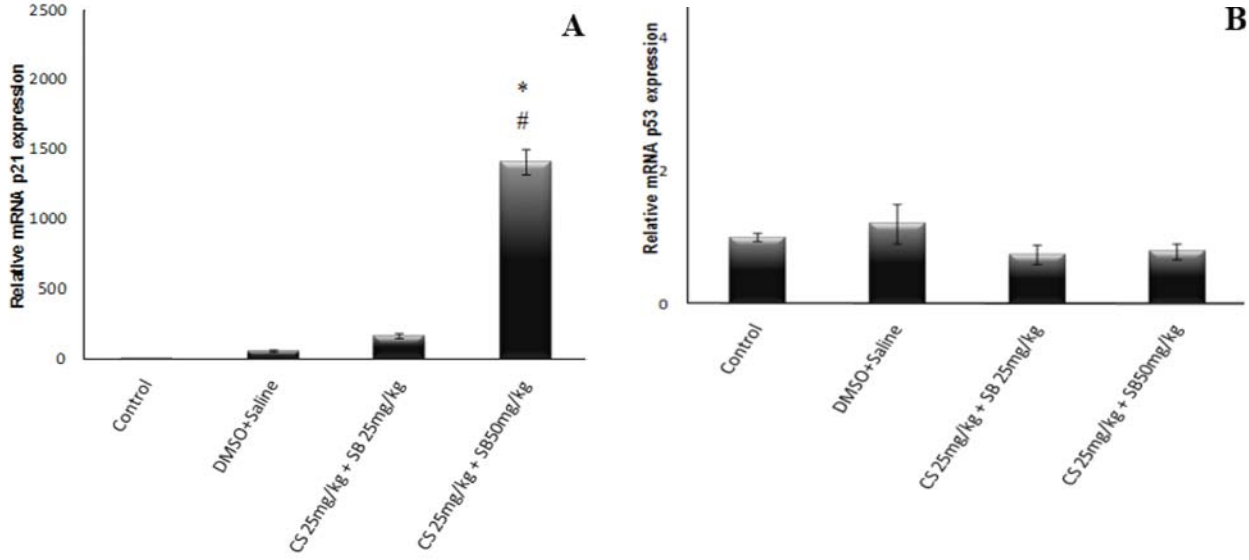

Figure 9. (A) Silibinin in combination with chitosan increases p21 expression in EAC mice model (25.3 fold at a dose of 50 mg/kg silibinin + 25 mg/kg chitosan) *Significant difference compared to DMSO + saline treated groups at $p < 0.001$. #Significant difference compared to silibinin at 25 mg/kg + chitosan at 25 mg/kg groups at $p < 0.001$. **(B)** Silibinin (at different doses 25 & 50 mg/kg) in combination with 25 mg/kg chitosan has no significant effect on p53 expression.

22.23 fold. At the same time, it showed no significant effect on p53 expression level, when compared to the vehicle group (DMSO) as shown in Figure 8 (A, B). It was also seen that EAC-DMSO treated group (vehicle group) showed no significant effect on the expression level of both p53 and p21 when compared to the EAC bearing mice non-treated group.

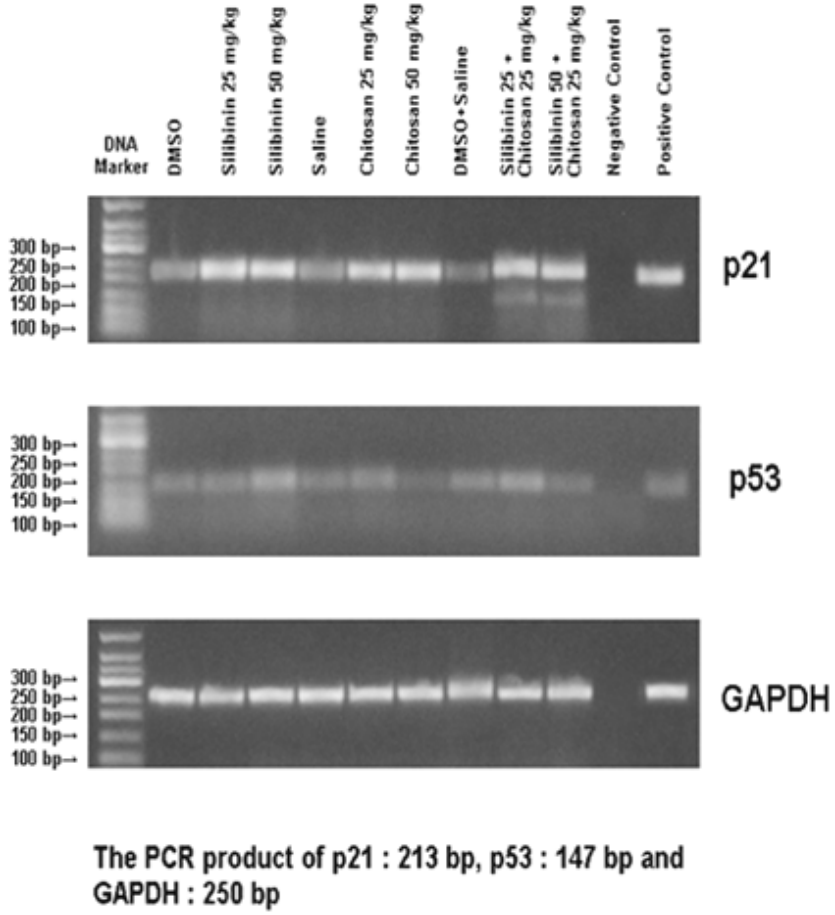

Figure 10. Final PCR product.

Effect of combinatorial treatment of CS and SB on the expression level of p53 and p21 genes

Figure 9A, B demonstrates that combination of CS at a dose of 25 mg/kg with SB at a dose of 25 mg/kg did not show significant increase in either p53 or p21 mRNA compared to the vehicle group (DMSO + saline). It also demonstrates that using CS at a dose of 25 mg/kg with SB at a dose of 50 mg/kg showed a significant increase in p21 gene expression as it increased by 25.3 fold ($p < 0.001$), while it showed nearly no effect on p53 expression level compared to the vehicle group (DMSO + saline). Figure 9 also shows that EAC bearing mice DMSO + Saline treated group (vehicle) showed no significant effect on both p53 and p21 mRNA compared to EAC bearing mice non-treated control group.

Figure 10 demonstrates the final PCR products which were confirmed by 1.2% agarose gel electrophoresis.

DISCUSSION

The study was performed to identify promising anticancer agent alone or in combination with other that would exhibit fewer side effects and augments chemotherapeutic action using reduced doses to get higher overall performance. Furthermore, it was to provide the first comparative biochemical assessment of CS and SB as antitumor and antioxidant agents in mice bearing EAC tumors with their effect on expression levels of p53 and p21 genes.

The growth of EAC cells which we presently used in the mouse model to evaluate the antitumor activity of the compounds under investigation, has been similarly used by Gupta *et al*., 2004 [21] to evaluate antitumor activity of *Bauhinia racemosa* against EAC in Swiss albino mice. More recently, El-Far *et al.* [6, 7] used these EAC bearing mice as tumor model to evaluate antitumor activity of CS, carboxymethyl chitosan (CMC) and novel

water-soluble CMC-based co-polymer. They demonstrated that ascetic tumor volume expressed as mL can be used as a measure of antitumor effect in mice. Our data demonstrated that saline alone showed no antitumor activity, but DMSO, alone or in combination with saline, showed antitumor activity in the EAC-bearing mice when compared with their EAC-non treated control group. DMSO has been used in treatments of certain types of tumors as reported by other investigators [22]. Therefore, a possible mechanism underlying the inhibitory effect of DMSO on EAC cells could be due to its known action as a potent scavenger of hydroxyl radicals and reactive oxygen species (ROS) as well as peroxynitrite [23]. This would be in agreement with our present findings. This also shows the importance of using control groups containing the vehicle used.

We herein show that CS at a dose level of 25 mg/kg and 50 mg/kg showed a significant decrease in tumor volume at the end of *in vivo* experiment when compared to their EAC control group at the same experimental conditions. Furthermore, our results revealed that reduction of tumor volume, as a measure of antitumor effect, using CS is dose-dependent as 50 mg/kg dose exhibited more dramatic decrease than that observed with 25 mg/kg. Similar observation which supports this was recently reported by El far *et al.* [7], in their experiments with the same animal model but using two millions of cells for tumor induction. Other investigators [5, 24] have previously reported the antitumor effect of low molecular weight and oligomeric Chitosans. The advantage of the present CS study, in this regard, is due to the use of safe injectable water soluble solution of CS. It is well known that original CS is insoluble in neutral and alkaline pH conditions. In pH < 7, free amino groups are protonated, and the polysaccharide becomes soluble. So, the use of this allowed us to avoid making chemical modifications to improve its solubility. Qin *et al.*, [25], showed that antitumor activity of CS results from a simple change in its chemical structure, low molecular weight, water solubility and the degree of acetylation. So we have used medium molecular weight, which showed a significant antitumor effect and a decrease in tumor volume by more than 70% at a moderate safe dose level of 50 mg/kg, which was not previously reported. We show herein, for the first time, that SB at dose levels of 25 mg/kg, 50 mg/kg and 75 mg/kg injected to EAC bearing mice which received one million of cells for tumor induction, showed a significant decrease in tumor volume and consequently significant antitumor activity. This reduction is also dose-dependent compared to their control group at the same experimental conditions (Figure 11). As apparent, SB at a dose level of 75 mg/kg, showed a complete disappearance of tumors in all treated animals with a single exception of one animal which showed partial significant response. Although recent studies have provided evidence of antitumor activity of SB [26, 27], we reported for the first time, complete response to SB treatment in about 88% of the total number of animals under investigation. Indeed, SB at a dose level of 75 mg/kg, when administrated alone, displayed the strongest antitumor activity, compared to other studied groups. In the current research, we presented the first-time investigation that looked into the antitumor activity of SB either alone or in combination with CS using EAC bearing mice which received one million of cells for tumor induction. Our results indicate that the combination of CS at dose of 25 mg/kg with SB at dose 50 mg/kg showed complete response to treatment in about 67% of the total animals, and this elicited the second highest antitumor activity in our protocol (Figure 12). The additive effect cannot be excluded, as SB alone at dose level of 50 mg/kg showed complete response in about 54%, while CS alone at dose levels of 25 mg/kg showed only 23% complete response to treatment. Another evidence which shows that SB dose is the key factor for such additive effect can be concluded from the fact that combinatorial treatment using SB (50 mg/kg) with CS (25 mg/kg) showed higher antitumor activity when compared with combinatorial treatment using SB (25 mg/kg) with CS (25 mg/kg); the former group showed complete response to treatment by about 67%, while the second group showed about 17% complete response, of the total number of animals used in each group.

Previous studies indicated that the progress of tumor in EAC bearing mice is reflected by the increased ascetic tumor volume as well as increased number of viable tumor cell count in it [7, 21]. This is in agreement with our present findings. It is well known that the presence of oxygen is very important and vital to our life, but

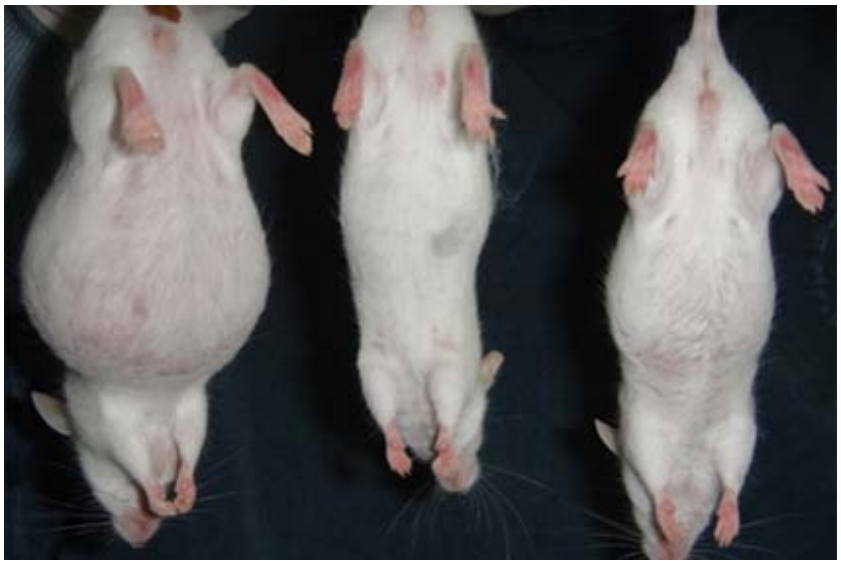

Figure 11. Effect of Silibinin (SB) at dose of 50 mg/kg on mice with Ehrlich ascites carcinoma (EAC) (right) and (SB) at dose of 75 mg/kg on mice with EAC (middle) compared to an EAC control animal (left). Substantial difference observed on treated animals.

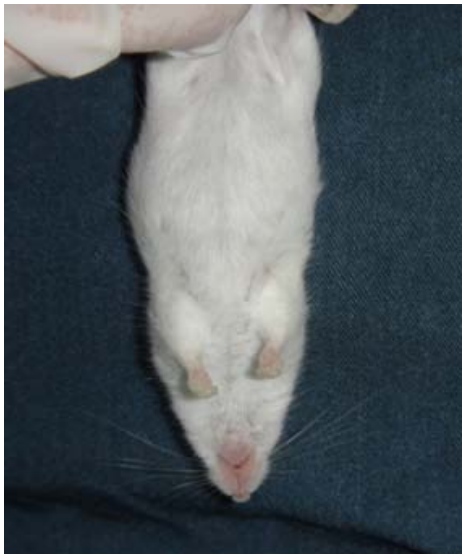

Figure 12. Effect of combinatorial treatment of Chitosan (CS) and Silibinin (SB) at doses of 25 and 50 mg/kg, respectively on mice with Ehrlich ascites carcinoma (EAC).

this gas during metabolic utilization may result, under certain conditions, in the production of some undesirable by-product named as superoxide, a reactive oxygen species (ROS). A review by Valdivia *et al*., [28] extensively discussed the central role of this superoxide when present in excess to cause several diseases, including cancer. They extensively showed how it is cleared successfully by SOD. As apparent from our data the administration of CS, SB or their combination have significantly increased SOD activity in treated groups, indicating the antioxidant and free radical scavenging property of both CS and SB. SB (50 mg/kg) showed highest increased SOD activity by 73% compared to control group. Also, administration of CS alone (25 mg/kg) or SB alone (25 mg/kg), or in combination using the same doses have elicited the second highest SOD activity compared to their control groups. They were found to be within the same range. This clearly demonstrates the potential use of CS, SB or their combination as an inhibitor of EAC induced intracellular oxidative stress due to superoxide, as it is well known that the excessive production of free radicals cause damage of macromolecules such as lipids which induces lipid peroxidation *in vivo* [29, 30]. So, following treatments using CS and SB either alone or in combination, can restore SOD activity and able to correct and reverse the imbalance between ROS and antioxidant defense by restoring and augmenting the antioxidants status due to elevation of SOD activity. SOD is well known as the first line of defense against oxygen toxicity as it catalyzes the dismutation of

superoxide anion producing hydrogen peroxide, reducing the dangerous presence of $O_2^{-\bullet}$, and thus prevents its harmful actions to cells and normal organs and consequently participates in inhibiting the initiation as well as promotion stages of carcinogenesis. The antioxidant and free radical scavenging characteristics of CS and SB were previously reported by others [5, 31, 32], which are in agreement with the results of this work. Our result in this regard provide another evidence and clues on the mechanism through which antioxidant enzyme SOD may influence tumor growth after administration of CS, SB or their combination. The significant increased activity of SOD in target tumor cells of treated mice with different agents under investigation in the present study, concurrently with a significant decrease in tumor volume as well as number of viable cells, in the same group of animals, would clearly provide evidence of restoring and augmenting antioxidant defense enzyme SOD after treatment. It has been shown that SB increased SOD expression in human HCC xenografts in nude mice [33]. This supports our results and postulation. To the best of our knowledge, this is the first study to compare the effects of CS and SB or their combination using the same experimental conditions. Other studies demonstrated that administration of CS increased cellular SOD activity in tumor cells in experimental animal models [7]. No previous reports indicated the dual action of using combination of CS and SB on SOD activity in tumor cells, as reported here. We showed, for the first time that administration of SB increases SOD activities in a dose-dependent manner, which clearly indicate the strong antioxidant and free radical scavenging property of SB which in turn is partly responsible for its potent antitumor activity as we demonstrated. The chemopreventation effect of silymarin as a strong antioxidant was found to work via protection of the membrane from ROS toxicity, thus inhibiting the initiation as well as promotion stages of carcinogenesis [34]. As SB is one of the main constituents of silymarin, such mechanism cannot be excluded for the action of SB as chemopreventive agent.

MDA is a short-chain aldehyde and when present in excess it leads to oxidative damage in cell membrane, lipoproteins and other lipid containing structures, and thus MDA is used as biomarker of lipid oxidation and oxidative stress [35]. MDA, was reported to be higher in carcinomatous tissue than in non disease organs [36]. Furthermore, Kaynar *et al.,* [37] indicated that increased lipid peroxidation in abnormally proliferating cells leads to an increase in serum MDA of patients with cancer. In the present study, treatment with SB alone at a dose level of 25 mg/kg and 50 mg/kg significantly reduce cellular lipid peroxide MDA level compared to their control group. Also, treatment with SB in combination with CS using low dose level of both, i.e. 25 mg/kg, also showed decreased MDA level by 40% compared to their control group. The decrease in MDA level in tumor target cells along with the significant decrease in tumor volume in same groups, suggest a positive association between the antitumor activity of SB and its antioxidant activity as well as anti-lipid peroxidation properties. This preposition is substantiated and explained by the work of Gazak *et al.*, and Ligeret *et al.,* [38, 39] which revealed a marked reduction in oxidative stress and lipid peroxidation by SB treatment. Also, the increase in lipid peroxidation in cancer was attributed to be due to poor antioxidant system [40], and it has been claimed that MDA acts as a tumor promoter and co-carcinogenic agent because of its high cytotoxicity and inhibitory action on protective enzymes [41, 42]. This would explain our present findings and is also in agreement with it. Furthermore, a simultaneous inhibitory effect of SB on lipid peroxidation by significantly decreased MDA production as well as high antitumor activity that we reported, may partly depends also on its strong SOD activity, since it has been suggested that $O_2^{-\bullet}$ participates in the initiation of lipid peroxidation reaction and production of MDA [43, 44], as well as in causing cancer [28]. Thus, we suggest that SB exert a protective effect against lipid peroxidation as a free-radical scavenger agent.

The role of NO in tumor biology is complex as NO may play a dual role in tumor progression, as it can act as promoter or antitumor promoter, depending upon its concentration as reported by Eijan *et al.*, [45]. Taysi *et al.,* [46] showed that the concentration of NO, under non pathological conditions was found to be in the nanomolar range, but under conditions of oxidant injury it was found to be in the micromolar range. That is why we studied the changes of NO levels in tumor target cells after treatment using CS, SB or combinatorial treatment of both in EAC-bearing mice. Previous studies

showed that EAC inoculation induced significant increase in the serum MDA and NO levels compared to that of the normal control group [47]. Current results revealed that administration of SB at a dose level of 50 mg/kg produced significant depletion of NO level by 70% compared to that of EAC control group, while at dose of 25 mg/kg exhibited a decreased level of NO by 51.5%. This shows that it acts in a dose dependent manner. This significant decrease in NO level, along with the observed decrease in tumor volume by treatment with SB at different doses, suggests a positive association between the antitumor activity of SB and NO levels. Simply, higher dose of SB increase significantly the tumor growth inhibition. This is in agreement with previous reports which showed that NO levels were significantly increased in tumor tissues of patients with colon cancer [48]; others showed also that erythrocytes NO levels were significantly higher in patients with small cell and non-small cell lung cancer than in controls [37]. Our results are in agreement with those of others [49], who reported that elevated level of lipid peroxidation and its product MDA can stimulate host cells, mainly monocytes/macrophages, to produce and release NO by the induction of inducible nitric oxide synthetase (iNOS) activity resulting in tissue as well as DNA damage. Our present results also showed that SB treatment with different doses produced a simultaneous significant decrease in cellular MDA level along side a significant decrease of NO levels and increase of SOD activities in tumor target tissue. This could be explained in view of the work of Kaynar *et al.*, [37] who also demonstrated the positive correlation between erythrocyte MDA level with erythrocyte NO levels in patients with early stage non-small cell lung cancer. Concerning association between SOD activity and NO level, SOD found to be intimately involved in the regulation of $O_2^{-\bullet}$, and the metabolites of nitrogen [50], as $O_2^{-\bullet}$ when combines and reacts rapidly with NO forms peroxynitrite (ONOO-) [51]. Peroxynitrite is cytotoxic and much more reactive than NO and $O_2^{-\bullet}$ and causing different chemical reactions in biological system including nitration of tyrosine residues of proteins, triggering of lipid peroxidation, which inhibits the mitochondrial electron transport, and oxidation of biological thiol compounds [47, 52]. So, it is clear that NO concentration plays a role in tumor biology, and treatment with silymarin was found to suppress iNOS gene expression [53], which demonstrated that silymarin causes a strong protective effect against carcinogenesis via down regulation of inflammatory and angiogenic responses, including iNOS. This supports our postulation that SB reduces NO production mainly via inhibition of iNOS activity and partly via increasing NO clearance, as SB is one of the major constituents of silymarin. This is also based upon the work reviewed recently by Ting *et al.* and Singh *et al.* [15, 54]. On the other hand, the effect of CS on MDA and NO levels in EAC bearing mice showed no significant change in the values and were found, in the present study, to be within normal ranges. This tends to indicate the absence of any harmful lipid peroxidation or nitrosative stress upon using CS. This is in agreement with recent findings in [7] and others. This action of CS on lipid peroxidation is different from that observed for SB, as CS treatment has led to an augmentation and significant increase in cellular SOD activity in EAC bearing mice, causing augmentation of the antioxidant defense system, but CS does not affect significantly the levels of NO or MDA at the dose level used in the present investigation, which indicate no trends in causing oxidative stress. In the present study, we also noticed that low dose of CS (25 mg/kg) produced no effect on MDA level, while CS at dose level of 50 mg/kg exhibited decreased MDA by nearly 30% compared to EAC-control group. This suggests that suppressive action of CS on MDA level and lipid peroxidation depend on its dose level. Interestingly, it has been shown that low molecular weight and higher concentration of chitosan has a positive influence on the antioxidant activity. Conversely, a higher molecular weight was found to be the most effective factor in reducing lipid peroxidation as reviewed by [5]. That is why we used medium molecular weight of CS in our studies, and that might explain why different results may be obtained using different molecular weights or concentrations of CS. To the best of our knowledge, our present work using medium molecular weight of CS is the first to be used in a comparative biochemical assessment of CS and SB as antitumor agents and potential antioxidants.

Kuppusamy and Karuppaiah, 2012 [55] recently demonstrated that the oral administration of CS at

a dose level of 100 mg/kg significantly suppressed the lipid peroxidation formation in bladder tissues of animals bearing bladder cancer induced by benzidine, which is not only in agreement with our present results using EAC-bearing animals, but also supports it. The suppressive action of CS on lipid peroxidation formation was believed to be due to the ability of CS to make a direct effect on the cell membranes and may thus decrease the susceptibility of the membranes to lipid peroxides. In the present study, CS treatment alone at high-dose level or in combination with SB led to the augmentation on the antioxidant defense through significant increase in SOD activity as an antioxidant enzymatic biomarker, concurrently with improvement in levels of oxidative stress biomarker (MDA). This dual action can correct and reverse the imbalance between ROS and antioxidant defense after treatment with both agents alone or in combination with each other, which in turns increase their antitumor activities in EAC-bearing mice. Recent review showed that antioxidant activity of compounds has been attributed to various mechanisms such as the prevention of chain initiation, the binding of transition metal ion catalysts, the decomposition of peroxides, a reductive capacity and radical scavenging [5]. They showed that the scavenging activity of CS is due to its strong hydrogen-donating ability; ROS can react with active hydrogen atoms in hydroxyl or amino groups of CS to form a very stable macromolecular radical. This review shows that different studies investigated CS as a potential antioxidant agent. On the other hand, another recent review article by Tan *et al.*, [56] has extensively discussed the effect of SB on many cancers, the potential of SB as antioxidant agent and its mechanism(s) of action as an anticancer agent. The combined use of CS with SB, we used for the first time, suggesting the therapeutic potential of this combination and deserves more investigations in the future.

We conclude that SB at a dose level of 75 mg/kg showed superiority as potent antitumor agent and powerful antioxidant, over other treatments under investigation either using SB at other different doses alone, or in combination with CS at different doses. However, future investigations are still needed in this promising agent alone or in combination with other modalities or compounds. Evidence for well-tolerated administration of high doses of silibinin in human patients without noticeable toxicity was provided [57, 58] Our present data showed that administration of SB at a dose level of 75 mg/kg showed 100% survival rate. This could be explained in view of the work provided by others [59], who indicated that SB not only inhibits primary prostate tumor progress but also protects against angiogenesis and late-stage metastasis. Therefore, SB may have a potential for improving survival and reducing morbidity in cancer. The mortality rate as expressed by survival percentage was evaluated in the present study as a reliable criteria for judging the value of any anticancer agent which depends on the survival rate after treatment [60]. This supports our observation in the present study which showed that i.p. administration of CS and SB exhibited no toxicity or any abnormal behavioral responses in treated animals under investigation and doses presented.

Our results also clearly demonstrate that SB upregulated p21 gene expression which is a CDKI in a dose-dependent manner, and at the same time it had no significant effect on P53 gene expression in tumor cells of EAC model. This suggests that upregulation of p21 is p53-independent after using SB, especially at higher dose. Our results are in agreement with other studies [61, 62], as they showed that the upregulation of p21 is a p53-independent mechanism. Upregulation of P21 has been reported by other investigators [63, 64], but in different cancer models, as they demonstrated that SB potently inhibited the growth of HT-29 and LoVo cells both *in vitro* as well as in xenograft models. This inhibitory effect is due to a lot of mechanisms, including induction of G1 and more modestly G2-M cell cycle arrest and increased levels of CDKIs (Cip1/21 and Kip1/p27). So, the antitumor effect of SB we reported appears to be correlated to significant increase in p21 gene expression which is correlated with cell cycle arrest without showing significant effect on p53 gene expression. It is well believed that cancer develops when the balance between cell proliferation and cell death is disrupted, and the ensuing aberrant proliferation leads to tumor growth. Furthermore, the cyclin-dependent kinase inhibitor p21, found to be induced by both p53-dependent and -independent mechanisms following stress and induction of p21 may cause cell cycle arrest.

So p21 as a proliferation inhibitor is poised to play an important role in preventing and treating tumor development [65]. It has been postulated that SB exerts its anti-cancer activity mainly by blocking cell cycle progression and induction of G1 cell cycle arrest in a dose- and time-dependent manner in large cell carcinoma H1299 and H460 cells and bronchioalveolar carcinoma H322 cells [66]. This supports our *in vivo* present results which show antitumor SB effect in a dose-dependent manner. SB is also found to modulate the protein levels of cyclin-dependent kinases (CDKs; 4, 6 and 2), cyclins (D1, D3 and E), and CDK inhibitors (p18/INK4C, p21/Waf1 and p27/Kip1) in a differential manner in the different cell lines [66].

Our results clearly demonstrate that CS at a lower dose (25 mg/kg) shows a significant upregulation of p21 gene expression, which is a CDKI, as it increases by 3.07 fold and at the same time has no significant effect on P53 gene expression in tumor cells of EAC model. While the higher dose of CS (50 mg/kg) shows no significant effect on both p21 and p53 gene expression when compared to their vehicle control group, indicating that the upregulation of p21 is a non-dose dependent. These results clearly demonstrate that CS treatment resulted in tumor growth inhibition through increasing p21 mRNA independently of p53, which leads to cell cycle arrest. It also exerts its antitumor activity by its antioxidant effect as discussed. Many researchers have reported that the growth-inhibitory effect of CS on human bladder tumor cells can be attributed to apoptosis induction [67]. This may confirm our results. Other mechanisms were presented by Lee *et al.* [68] who showed that CS is a kind of cationic polymer, and the surface charge of CS and its derivatives is the major factor affecting its cytotoxic activity due to the electrostatic ionic interaction between the negatively charged groups of tumor cells and the positively charged amino groups of chitosans [68].

Our studies showed that expression level of p21 and p53 showed no significant increase after using the combination of CS 25 mg/kg + SB 25 mg/kg. On the other hand, the use of the combination CS 25 mg/kg + SB 50 mg/kg showed an extremely significant increase in p21 mRNA, while this combination had no significant effect on p53 expression level. These data showed that the upregulation of p21 is dose dependent, and the key factor is the increase in the dose of SB. This conclusion is in agreement with the effect of SB on p21 mRNA at different doses. Our data has revealed for the first time that the combination of CS at a dose of 25 mg/kg with SB at a dose of 50 mg/kg had an additive effect on p21 expression level by 25.3 fold when compared with CS (25 mg/kg) alone and SB (50 mg/kg) alone as they upregulated p21 mRNA by 3.07 fold and 22.23 fold, respectively. This also suggests a positive association between the antitumor activity and p21 upregulation, as in fact the combination treatment (CS 25 mg/kg + SB 50 mg/kg) also showed significant anticancer effect in our presented data, which is also superior over CS. These results mean that these combinations upregulate p21 in a p53-independent manner and this is in agreement with previous studies done by Russo *et al.*, [69] who also demonstrated that p21 overexpression leads to a dual outcome, activating G1/S arrest of the cell cycle or the apoptotic pathway through mitochondria, depending on its intracellular levels. It is noteworthy that depletion of p21 abrogates both effects.

CONCLUSION

We suggest that using a novel combination of naturally derived compounds (CS and SB) or using SB alone might be a potential candidate for developing multifunctional anti-cancer agent; however, these treatment modalities deserve more investigations in the future.

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